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A comparison of life-history traits in calcifying Spirorbinae polychaetes living along natural pH gradients

Running head: Identifying traits tolerant to low pH

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Abstract

Low pH vent systems are ideal natural laboratories to study the consequences of long-term low pH exposure on marine species, and thus identify life-history traits associated with low pH tolerance. This knowledge can help to inform predictions on which types of species may be less vulnerable in future ocean acidification (OA) scenarios. Accordingly, we investigated how traits of calcifying polychaete species (Serpulidae, Spirorbinae) varied with pH using a functional trait analysis at two natural pH gradients around Castello Islet in Ischia, Italy. We first observed the distribution and abundance patterns of all calcifying polychaete epiphytes in the canopy of *Posidonia oceanica* seagrass across these gradients. We then used laboratory trials to compare fecundity, settlement success, and juvenile survival in the dominant species from a control (*Pileolaria militaris* Claparède, 1870) and a low pH site (*Simplaria* sp., (Serpulidae, Spirorbinae). We found significantly higher reproductive output, juvenile settlement rates, and juvenile survival in *Simplaria* sp. individuals from the low pH site, compared to *P. militaris* individuals from control pH sites, when observed in their respective *in situ* pH conditions. Our results suggest that tolerance to low pH may result, in part, from traits associated with successful reproduction and rapid settlement under low pH conditions. This finding implies that other species with similar life history traits may cope better in future OA scenarios, and should be targeted for future OA tolerance research.

Keywords: Ocean Acidification, Calcifiers, Settlement Success, Fecundity, Early-life Survival, Serpulidae, Population Resilience

1 Introduction

Anthropogenically-driven global changes may reduce or alter marine biodiversity (Raven et al. 2005, Widdicombe & Spicer 2008). One such change, ocean acidification (OA), occurs when surface seawater absorbs increasing atmospheric carbon dioxide (CO_2), resulting in lowered pH and reduced availability of the carbonate ions many marine organisms require to build skeletal structures (IPCC, 2014). Despite confidence in forecasts of the chemical impacts from this process into the next century (Bopp et al. 2013, IPCC 2014), uncertainty surrounds the corresponding biological and ecological impacts (Harley 2011, Gaylord et al. 2014). Determining sensitivities and tolerances to future OA conditions represents a necessary first step in improved understanding of how marine biodiversity will change in the next decades (Stockwell et al. 2003, van Oppen et al. 2015).

Functional trait analyses (FTA) can help determine sensitivity or tolerance of different species to low pH conditions. These analyses link environmental gradient survey data (e.g. species abundance patterns), to specific phenotypic trait changes in two or more species along the same gradients (e.g. body size, reproductive habit, fecundity) (McGill et al. 2006). The assumption is that by comparing a specific trait among phylogenetically similar species (i.e. sister species, or species derived from a common ancestral node), differences can be attributed to specific environmental effects. In the context of future OA, application of specific functional trait analyses along natural pH gradients provides a relatively straightforward tool to evaluate which types of species will play pivotal roles in reorganizing the biodiversity landscape (McGill et al. 2006).

Abundance surveys performed in volcanic vent systems, which emit CO_2, have recently proven useful in identifying traits associated with invertebrate species sensitivities and tolerances to future OA change (Lucey et al. 2015, Gambi et al. 2016). For example, marine invertebrates without a pelagic life stage (i.e. brooders or direct developers) are more abundant in low pH sections of CO_2 vent gradients (Lucey et al. 2015). Identification of a specific brooding oyster species with increased survivability in a low pH upwelling environment (Waldbusser et al. 2016) reinforced this pattern Unfortunately, natural system assessments generally lack trait data regarding fecundity, larval survival, settlement and recruitment (however, see Padilla-Gamino et al. (2016) for coralline algae assessment). This gap partly exists because field observations cannot provide detailed data involving a temporal element (e.g. settlement rates), or observations requiring thorough quantification of small-scale processes (e.g. egg production, fecundity). Collecting and observing such traits in a laboratory setting that mimics the individuals' *in situ* conditions can help to fill this gap. Combining such laboratory trials with natural system-based assessments may offer one mechanism to strengthen analyses of some functional traits.

The sensitivity of early developmental stages underscores the need to understand marine invertebrate life histories within future OA scenarios (Kurihara 2008, Albright 2011, Byrne 2011, Crook et al. 2016). As such, our study aims to identify fecundity and early life history traits associated with low pH tolerance in marine invertebrates using calcifying serpulid polychaetes (Spirorbidae, Annelida) along two natural pH gradients. These gradients border the Castello Aragonese Islet off the coast of Ischia (Naples, Italy), and are a formed when volcanically derived CO_2 gas bubbling up through the seafloor

mixes with seawater, decreasing the pH from an ambient value of ~8.17 to as low as 6.57 (Tedesco 1996, Hall-Spencer et al. 2008, Kroeker et al. 2011, Garrard et al. 2014), well representing business-as-usual IPCC pH projections for 2100 (IPCC, 2014) .

We selected calcareous polychaetes of the sub-family Spirorbinae (Annelida, Serpulidae), which inhabit *Posidonia oceanic* seagrass leaves as epiphytes along the Castello pH gradients, as our study focus. Generally, Spirorbinae polychaetes encompass a diverse suite of life history traits that can vary among closely related species (Macdonald 2003). Their dual life stages, with both free-swimming larval phases and sessile adult stages (Kupriyanova et al. 2001, 2006), offer another beneficial attribute.

First, we assess the distribution and abundance patterns of the calcifying polychaete assemblage on *Posidonia oceanica* leaves across two natural pH gradients bordering the Castello Islet: – one along the north (mean pH range 7.39 - 8.03), and one along the south (mean pH range 6.99 -8.03). Second, we use laboratory trials comparing two closely related species - one from a low pH site and the other from a control pH site - to investigate possible links between fecundity, juvenile survival, settlement traits, and low pH tolerance. Knowing which of these life history traits are associated with low pH tolerance will help inform our predictions on the types of species that may be less vulnerable in future ocean acidification scenarios

2 Materials & Methods

2.1 Field survey

106 The pH gradients used in this study occur along the north and south sides of the
107 Castello Aragonese islet on Ischia, an island off the coast of Naples, Italy (Tyrrhenian
108 Sea) (Fig. 1). To represent three pH levels, six sampling sites were chosen within
109 *Posidonia oceanica* seagrass meadows, at depths of approximately 3 ± 0.5 m. Sites are
110 referred to as N3, N2, NC and S3, S2, SC, where "3" represents the extreme-low pH, "2"
111 the low pH conditions, and "C" the control pH. The "N" and "S" represent north and
112 south gradients (Fig. 1). Sites were chosen to be were comparable in depth and *Posidonia*
113 cover (Donnarumma et al. 2014). The site names are listed with their corresponding
114 carbonate seawater parameters in Table 1. Seawater parameters represent a synthesis of
115 all available data in the last six years to convey the most comprehensive and realistic
116 time-series data for these study sites (Ricevuto et al. 2014). For additional water
117 parameters and GPS coordinates, see Supplementary Material: *Site Details*.

118 To determine which calcifying polychaete species settle on *Posidonia* leaves in
119 low pH environments, and how their abundance and distribution varies along the pH
120 gradients, sampling by SCUBA diving was performed on September 29th - 30th, 2014.
121 Four quadrats (replicates) of 40 x 40 cm were haphazardly placed at least 2 m apart on
122 the seagrass canopy in each pH site. Within each quadrat, leaves of ten *Posidonia* shoots
123 were randomly cut at the base of the rhizome and put in separate plastic bags. In the two
124 extreme low pH sites (N3 and S3), initial visual inspection showed a highly reduced
125 number of worms on leaves. Consequently, the number of sampled shoots was increased
126 by cutting only the external leaf (oldest leaf) of 30 shoots within each of the four quadrats
127 in both N3 and S3. This provided a more reliable estimate of worm abundance and helped
128 preserve the seagrass from impact due to sampling. Samples were transferred in bags

129 containing seawater to the Villa Dohrn-Benthic Ecology Center of Ischia (Stazione
130 Zoologica Anton Dohrn) within 1 h of sampling, and preserved in 4 % neutralized
131 formalin for 24 h. They were then rinsed with fresh water and transferred into 70 %
132 EtOH for long-term preservation.

133 The number of calcifying polychaetes on the *Posidonia* leaves of each shoot was
134 determined by viewing each leaf from each replicate/quadrat under a dissecting
135 microscope (AZ100, Nikon, Milan, Italy; magnification 1- 50x). Species were identified
136 from their tube orientation, operculum and chaetae morphology. Due to the loss of some
137 opercula, some specimens remained unidentified. These were included in the counts by
138 determining the ratio between the number of specimens identified for a given species and
139 the total number of specimens found at each site. This ratio was used to calculate the total
140 number of each species for each site replicate (Supplementary Materials: *Table S.1*).

141 The average number of polychaetes at each site accounting for differences in the
142 available settlement area was calculated by multiplying the shoot density to the
143 settlement area (percentage of *Posidonia* shoots colonized by spirorbids * average
144 number of spirorbids *per* shoot), with shoot density data from Donnarumma et al. (2014).
145 Only leaves longer than 5 cm were considered. In the extreme low pH sites (S3 and N3),
146 where sampling included only external leaves, the estimation followed the same
147 procedure.

148 2.2 Laboratory trials

149 One day after the field survey, live individuals were collected for the laboratory
150 trial by SCUBA diving. This entailed cutting *Posidonia* leaves with visibly attached

polychaete tubes from the S2 (low pH) and NC (control pH) sites. Leaves were placed in fabric bags, keeping the individuals from both sites separated and in their original seawater conditions.

All material was transported to the ENEA Laboratory in La Spezia, Italy where specimens were sorted, identified and prepared for the trial (Supplementary Material: *Transport Details*). Specimens were held at the pH conditions from their respective field sites (control or low pH) during the sorting process (2 – 4 d). For the trial, we identified 18 *Pileolaria militaris* adults from the control pH site (NC), and 12 *Simplaria* sp. adults from the low pH site (S2).

These sites were chosen because they have the greatest average pH difference (Table 1), and specific species were chosen because the low pH site's sample was dominated by *Simplaria* sp. and the control pH site's sample was dominated by *P. militaris*. Additionally, the lack of *Simplaria* sp. individuals found in the control pH sites, and of *Pileolaria militaris* individuals found in the low pH sites, precluded a reciprocal laboratory transplant experiment. This in itself demonstrates that different species have different levels of sensitivity to low pH conditions, and thus the comparison in life history traits between the two selected species represents the best avenue to identify traits that help defining species' successful colonization in low pH. As in most classical functional trait analyses, this between-species comparison is able to capture valuable information.

At the start of the trial, each adult was placed in a separate Petri dish preconditioned with a biofilm from a 2 d non-filtered seawater soak and filled with 3 mL

of pH-conditioned filtered seawater (1 adult *per* Petri dish). The pH in the dishes was set at 7.61 for *Simplaria* sp. adults, representing the average value found in the S2 field site considering time-series data (Table 1). Similarly, dishes with *P. militaris* adults were maintained at the control pH value, 8.1. All other seawater parameters matched the field values for both species (Table 2). In this setup, eight covered aquaria were filled with 20 mL of seawater. This water served as a bath for the uncovered Petri dishes (6-8 dishes *per* aquaria). Half of the aquaria were maintained at the lowered pH level by bubbling enriched (elevated pCO_2 air) into the seawater, while the other half was maintained at the control pH level by bubbling normal air into the seawater. The pH inside each Petri dish was attained through surface CO_2 diffusion within the covered aquaria (Gattuso 2011). The pCO_2 going to these aquaria was measured continuously throughout the exposure period using a CO_2 gas analyzer (Li-820, Li-Cor Biosciences, Lincoln, NE, USA). All aquaria were held in a thermal water bath that maintained stable thermal conditions. Petri dishes were randomly moved between the aquaria every two days.

Seawater pH, temperature, and salinity were measured in each Petri dish daily with an integrated pH and temperature meter (SG2, Italy), and refractomer (V2, TMC, São Julião do Tojal, Portugal). The pH meter was calibrated daily with pH buffer standards (4.01, 7.0, 9.21; Mettler-Toledo, Leicester, UK). Seawater samples (250 mL) were taken at the beginning and end of the trial from the stock seawater prepared for each treatment. Samples were fixed with $HgCl_2$ (0.02 %) to eliminate microbial activity, stored in borosilicate flasks (250 mL), and maintained in dark, dry conditions until total alkalinity (A_T) was determined using gran titration method (Dickson et al. 2007). Carbonate-system parameters of pCO_2 (μatm), total carbon dioxide (TCO_2, mol kg^{-1}),

196 bicarbonate concentration (HCO_3^- mol kg^{-1}), calcite saturation (Ω_{ca}), and aragonite
197 saturation (Ω_{ara}) were calculated from A_T, pH_T (total scale), temperature and salinity
198 using the package SeaCarb v.2.4.8 in software R (Lavigne & Gattuso 2013). Water-
199 chemistry parameters for each dish during the 14 d experimental phase, as well as
200 discreet field data from each pH site are presented in Table 2.

201 Seawater in each Petri dish was changed every other day by removing water with
202 a syringe and replacing it with new seawater. This water was collected from La Spezia
203 bay (La Spezia, Italy), and cleaned with a 0.1µm filter and UV sterilization system
204 (V2ecton 600, TMC, São Julião do Tojal, Portugal) for 5 d before being transferred to
205 sterile 2 L flasks. One flask was prepared for each treatment and placed in the
206 temperature bath described above with bubbling elevated pCO_2 air, or normal air,
207 depending on the treatment. Additionally, a diet of rotifers, *Artemia* sp. and microalgae
208 was added to seawater before each water change at 3 mL feed *per* 300 L seawater
209 (Gamma Nutraplus Reef Feed, TMC, São Julião do Tojal, Portugal). Petri dishes were
210 mixed three times *per* day by gently tilting aquaria to promote feeding. The density of
211 worms in each Petri dish was purposefully low to avoid potential indirect effects caused
212 by animal respiration (approx. < 0.092 mg^{-1}).

213 Under these laboratory conditions, all adults were monitored once a day with a
214 light microscope (AZ100, Nikon; magnification ranges of 25x up to 50x) for the presence
215 of embryos in the opercular brooding chamber. After adults released their first brood,
216 both adults and offspring were monitored daily for the following 14 d. The number of
217 offspring from each parent (brood size) was counted after the first day of brood release.
218 The number of settled larvae was counted daily, along with any deaths or additional

broods. The parent tubes were photographed with a digital camera (Nikon Sight DS-U1, Nikon, Milan, Italy) mounted on a light microscope (AZ100, Nikon), and tested as a trait covariate to account for any bias between parental size and offspring traits (i.e. brood size, mortality, brood survival). Photographs were analyzed with ImageJ software (Rasband WS, US National Institutes of Health, Bethesda, MD, USA) to obtain tube area (mm^2) (Abràmoff et al. 2004).

2.3 Data analysis

2.3.1 Field survey data

Two data sets generated from the field survey were analyzed: (a) the abundance of all calcifying polychaete species along the north and south pH gradients (distribution); (b) the abundance of the two dominant species, *Simplaria* sp. and *P. militaris* adults along the pH gradients. Initial data exploration using Cleveland dot- and boxplots revealed no outliers in either dataset. Conditional boxplots revealed heteroscedasticity of the variances among the pH sites for both datasets, and histograms indicated violation of normality (Züur et al. 2010a). Non-linear patterns within the species-level dataset also existed (Züur et al. 2010a). As a consequence, a Welch's ANOVA with a Games – Howell post-hoc test was used for both datasets to assess how the number of calcifying polychaetes varied along pH gradient, with 'gradient side' (north/south) and 'pH site' as fixed factors. This test is robust to non-parametric distribution of count data and heteroscedasticity of the variances.

Additionally, dataset (b) was analyzed by employing generalized additive models (GAMs) (Wood, 2006, 2011, 2014; Züur, 2010b) to describe the abundance of each species with respect to nominal 'pH' and to compare the abundance of each species along both gradient 'sides', GAMs accounted for the non-linear patterns in both the *Simplaria* sp. and *P. militaris* datasets, and were built using the mgcv (Wood, 2011) and nlme (Pinheiro et al. 2015) packages in R. For both species, gradient 'side' (factor: north or south) and 'pH' (fitted as a smoother) were set as the explanatory variables. Nominal mean water pH for each gradient side and site was based on the one-month average of September data from (Kroeker et al. 2011) to accurately represent seasonal pH values during the survey. The appropriate degrees of freedom of the smoothers were selected automatically using cross validation (Wood 2006, 2011). For *Simplaria* sp. only, the interaction between gradient side and pH was included using the 'by' command in the mgcv package (Wood, 2011). Both models were optimized by initially looking for the optimal random structure, followed by the optimal fixed structure (Züur et al. 2007). Akaike information criteria was used to compare models and residual plots were used to assess the mean-variance relationships; models for both species indicated no violation of the assumption for homogeneity of the variances. Over-dispersion was also calculated for each model (sum of Pearson residuals^2 / residual d.f.). High over-dispersion, particularly in *Simplaria* sp., required the use of negative binomial distribution with a log link (Pinheiro et al. 2015; Züur 2010b; Züur et al. 2007). The optimization function of the models (k parameter) was adjusted for this study's specific dataset at six. All statements about abundance change are based on the significance of the main effect gradient side, and not on the interaction between gradient side and pH.

2.3.2 Laboratory trials

In order to assess how fecundity and early life history traits differed in response to differing pH conditions, we compared responses of the low pH– originating *Simplaria* sp. adults (from, and dominant in S2) – under low pH conditions, to the control pH– originating *P. militaris* adults (from, and dominant in NC) – under control pH conditions with one-way ANOVA tests. Traits analyzed included: brood size of each parent; time of larval release to settlement (d); and percentage brood mortality *per* parent on d 7 and d 14. Data were tested for normality of distribution and homogeneity of variance using Cleveland dot- and boxplots. Boxplots indicated homogeneity of the variances among the pH species groups, and histograms indicated no violation of normality of distribution (Züur et al. 2010a).

All statistical analyses were performed by using the statistical software R (v.3.1.3; R Core Team 2015).

3 Results

3.1 Field survey

3.1.1 Species identity

All of the taxa found belonged to the Spirorbinae sub-family, within the Serpulidae family. The four main species were *Pileolaria militaris* Claparde, 1870, *Simplaria* sp., *Janua heterostropha* (Montagu, 1803) (= *J. (Dexiospira) pagenstecheri*), and *Neodexiospira pseudocorrugata* (Bush, 1905). A total of forty-eight undetermined Serpulinae were also encountered.

The taxonomy of the *Simplaria* sp. did not exactly match known records, but our primary prediction is that it is a putatively novel morphotype of *Simplaria pseudomilitaris* (Thiriot-Quievreux, 1965) – having more abundant, longer, and more pronounced distally projecting calcareous spines covering its operculum plate. Without further taxonomic analysis we reservedly designate these individuals as *Simplaria* sp.; and this inconsistency is further discussed in the Supplementary Materials: *Taxonomy Details*.

3.1.2 Species abundance and distribution

Total polychaete abundances on the *Posidonia* leaves along the pH gradient from the Castello CO_2 vents ranged from 0 to 224 individuals *per Posidonia* shoot. There was a decrease in the mean abundance from the control pH sites (SC and NC) to the extreme low pH sites (S3 and N3) along both the north and the south gradients, with a decline from 341 to 13 individuals in the south (SC to S3), and from 1,183 to 14 individuals in the north (NC to N3) ($F_{5, 92.97} = 75.11$, $p < 0.001$, Fig. 2A). The means in both the northern and southern extreme-low pH sites (N3 and S3) were comparable ($p > 0.05$, Fig. 2A). However, overall mean abundance was three times lower in the southern gradient compared to the north ($p < 0.05$, Fig. 2A). Additionally, in the north, there was a strong linear relationship between abundance and pH conditions. This differed from the south gradient, as mean abundance in the low pH site was highest (S2: 144 individuals), compared to the control pH site (SC: 124 individuals; $p > 0.05$, Fig. 2A). Higher shoot densities in the low pH sites compared to the control pH sites (Donnarumma et al. 2014) did not change the overall abundance patterns observed on both the north and south gradients (Fig. 3). For example, polychaete densities remained very scarce in the extreme

low pH sites despite a mean 1,000 shoots *per* m^2 in S3 compared to the mean 467 shoots *per* m^2 in SC, and 719 to 380 shoots *per* m^2 in N3 *vs.* NC.

The results also indicate that the two dominant species are *Simplaria* sp. and *P. militaris* within all the sites. These two species are also closely related to each other taxonomically, compared to the other species identified. This prompted separate analyses of the distributions of both *Simplaria* sp. and *P. militaris*. The results show that the distribution pattern seen along the southern gradient of the total species analysis is due to *Simplaria* sp. (Fig. 2B). Furthermore, in the species-specific analysis, the total abundance along the pH sites ranged from 0 to 498 individuals in *P. militaris* and from 48 to 532 individuals in *Simplaria* sp. While the overall number of individuals for both species was comparable, their distribution differed. As in the total species analysis, abundances significantly declined with decreasing pH when considering all sample sites (*P. militaris*: $F_{4, 11} = 9.37$, $p = 0.006$, *Simplaria* sp.: $F_{5,78} = 24.27$, $p < 0.001$ (Fig. 2B & 2C). The mean abundance of *P. militaris* was highest in the north compared to the south gradient (52 *vs.* 7, respectively), and decreased from the control pH to extreme low pH in the north (NC to N3), and low to extreme low in the south (S2 to S3). *Simplaria* sp. mean abundance was higher in the south than in the north gradient. The *Simplaria* sp. abundance in the low pH south site (S2) was not significantly different to the mean abundance in the south control pH site (SC), but was different in the north between the NC control and N3 extreme low pH site. Additionally, *Simplaria* sp. was the only spirorbid species found in the site with the lowest mean pH of the Castello vent system (S3 pH: 6.99 ± 0.34) (Hoffmann et al. 2011).

Comparisons of the smoothers (non-parametric curves) generated by the additive mixed models for the two gradients of both species confirmed that abundance decreases in both species with decreasing nominal pH across each gradient ($p < 0.001$ for both *P. militaris* and *Simplaria* sp., Fig. 4). For *P. militaris*, there were significant declines in abundance with decreasing pH along both north and south gradients, however the northern gradient had significantly more individuals compared to the southern gradient. In contrast, *Simplaria* sp. abundances in the north and south were not significantly different when pH values were greater than 7.9 within the gradient (Fig. 4B).

3.1.3 Laboratory trials

The laboratory trials revealed that life history trait values vary significantly along the pH gradients. The average number of offspring *per* brood from low pH– originating *Simplaria* sp. parents was significantly higher than from control pH– originating *P. militaris* parents: means 8.08 (± 1.54) *vs*. 3.61 (± 0.44) ($F_{1, 28} = 10.80$, $p = 0.003$, Fig. 5). Also, settlement success was significantly higher in *Simplaria* sp. compared to *P. militaris*: 86.5 % (± 6.8) compared to 13.4 % (± 6.3), respectively ($F_{1, 28} = 58.80$, $p < 0.001$, Fig. 5). Additionally, all offspring from the *Simplaria* sp. parents metamorphosed and settled within 1 h in low pH seawater, whereas less than 13 % of the offspring from *P. militaris* parents settled in the first 24 h in control conditions.

Juvenile mortality rates 7 d after the first brood release was 4.2 % (± 2.9) in *Simplaria* sp. and 48.8 % (± 8.6) in *P. militaris* ($F_{1, 28} = 16.77$, $p < 0.001$, Fig. 5). Net survival after 14 d, including additional offspring from subsequent broods, was significantly higher (6.3 times) in *Simplaria* sp. offspring with respect to *P. militaris* offspring*:* means 9.5 (± 1.7) *vs.* 1.5 (± 0.4) offspring *per* parent, respectively ($F_{1, 28} =$

352 26.90, $p < 0.001$, Fig. 5). Furthermore, between day 7 and day 14, 10 out of 12 parents
353 released a second brood in the *Simplaria* sp. group, but only 4 out of 18 parents from the
354 *P. militaris* group produced a second brood. No influence of parental tube size was found
355 on brood sizes ($p \geq 0.05$), and no parental mortality occurred during the 14 d trial.

356 **4 Discussion**

357 This study aimed to identify specific life history traits that offer species potential
358 advantages to tolerating future OA conditions. We identified two primary species along
359 the Castello pH gradients with a close phylogenetic relationship, *Pileolaria militaris* and
360 *Simplaria* sp., and found that the higher abundances of *Simplaria* sp. in low pH (S2) were
361 associated with the ability to produce more viable offspring able to quickly
362 metamorphose and settle in low pH conditions, compared to that of its close relative
363 dominant at a control pH site (NC), *P. militaris*, observed under control pH conditions.
364 Below we discuss potential physiological and ecological explanations for differences in
365 traits underling species' sensitivity to low pH.

366 *Physiology considerations*

367 The most noticeable finding was that rapid offspring development accompanied
368 OA tolerance, as demonstrated by the production of larvae that metamorphose in minutes
369 in the low pH– originating *Simplaria* sp. under low pH conditions, compared to the
370 multiple days required for control pH–originating *P. militaris* individuals in control
371 conditions. This is noteworthy because the challenges of calcification associated with
372 metamorphosis and initial juvenile tube growth in many marine invertebrates exposed to
373 OA conditions have been well documented, with demonstrated altered metamorphosis,

374 slowed juvenile growth, weakened juveniles' tubes, and tube dissolution under pH levels
375 comparable to those used here (Dupont et al. 2009, Byrne 2011, Lane et al. 2012). In
376 contrast, our results indicate that the low pH–originating *Simplaria* sp. appears to have
377 overcome these challenges. We hypothesize that this may be, in part, due to specialized
378 larval glands that are able to expedite the secretion of a primary tube, resulting in
379 successful metamorphosis. These specialized larval glands are commonly found in
380 Serpulidae species with lecithotrophic (non-feeding) larvae (Kupriyanova et al. 2001),
381 but while both of the species here have primary larval glands, there were noticeable
382 physiological differences them. The *Simplaria* sp. embryos and larvae had highly defined,
383 large glands compared to *P. militaris* (see Fig. 6; white spots in the *Simplaria* sp.
384 embryos). Moreover, in *Simplaria* spp. the contents of the primary shell gland are
385 extruded *via* the anus and the calcareous secretion is molded by the movements of the
386 larva into a tube capable of housing the entire settled larva in less than 5 min (Knight-
387 Jones, 1978) (Nott 1973, Potswald 1978, Beckwitt 1980, Qian 1999).

388 Another interesting finding was the increased adult fecundity of low pH–
389 originating *Simplaria* sp., compared to control pH–originating *P. militaris*. This was
390 despite no significant difference in opercular brood chamber size between the two species
391 (chamber size is directly proportional to the adult's overall size, and thus the number of
392 offspring produced *per* brood) (Kupriyanova et al. 2001). In general, both species
393 fertilize and incubate their eggs and embryos similarly: in a single chamber that provides
394 aeration and physical protection from the outside environment (Thorp 1975). When ready,
395 competent larvae exit these chambers through a pore at its base (Macdonald 2003).
396 Explaining the fecundity differences may therefore involve testing for improved internal

fertilization, and/or accelerated embryo incubation in the low pH-originating *Simplaria* sp., comparatively (Chaparro et al. 2008, Segura et al. 2010).

The fecundity differences may also be an outcome of plasticity from multi-generational exposure in the low pH–originating *Simplaria* sp. population (Rodríguez-Romero et al. 2015, Chakravarti et al. 2016). The possibility that plasticity may be the coping mechanism for species dealing with rapid changes has recently been revitalized, yet evidence of plasticity's role in promoting persistence is not consistent (Merilä 2015, Calosi et al. 2016). For example, a field-based reciprocal transplant experiment using *Simplaria* sp. collected from the same low pH site (S2) found that plasticity was not attributed to fecunditiy differences (Lucey et al. 2016). They also presented contradictory evidence that the low-pH originating *Simplaria* sp. were able to reproduce multiple times, in comparison to a population of control pH–originating *Simplaria* sp, yet inadequate sample sizes preventing statistical confirmation (Lucey et al. 2016). This alludes to the possibility that higher fecundity could be the consequence of modulating (i.e. plasticity) the 'number of broods over time.'

Rapid metamorphosis and increased fecundity was also coupled with lower offspring mortality during the first two weeks of offspring life in the low pH–originating *Simplaria* sp. group. This suggests that these *Simplaria* sp. will have a higher likelihood of recruitment success and overall population persistence, compared to the control pH–originating *P. militaris* group (Hunt & Scheibling 1997). The field survey supports this idea: *Simplaria* sp. adults with embryos were found at every site along the gradient regardless of pH.

The overall decline of *Simplaria* sp. individuals at extreme low pH alludes to a pH threshold. This in partial agreement with Saderne and Wahl, (2013), where growth rates and recruitment of spirorbid *Spirorbis spirorbis* individuals at extreme low pH/ high pCO_2 levels (3150 ± 446 μatm) were significantly reduced, whereas at more realistic pH levels for end of the century projections, individuals did not show any adverse effects (Saderne & Wahl 2013). These pH values closely correspond to the low (S2) and extreme low (S3) pH values in this study and corroborate the idea that each species has specific pH 'tipping' points, as demonstrated in the larval mussels' development, *Mytilus edulis* (Ventura et al. 2016). This theory that physiological tipping points may limit populations' pH tolerance complements that of Lucey et al. (2016), where abnormally low pH values at the low pH site (S2) may have confounded a potential local adaptation signature.

Ecological considerations

Predation may also be playing a role in the distribution of spirorbid species around the CO_2 vents, and their pH tolerance traits. Increases in spirorbids predation are likely as there have been documented increases in amphipod and copepod abundance in the low pH sites, known spirorbid predators (Knight-Jones et al. 1975, Kupriyanova et al. 2001 p.60). This helps explain the decrease in spirorbids at the extreme low pH sites. Furthermore, it suggests a potential correlation between increased predation and the novel opercular spine morphology observed in the low pH– originating *Simplaria* sp., where rows of long, slender calcareous spines project from the top of the operculum and guard the tube opening. Further investigation is necessary to prove this theory (e.g. Harris, 1968, Knight-Jones et al. 1974, Bianchi 1981; also see Supplemental Materials: *Taxonomy Details*). There are also indirect predation threats that may be influencing the spirorbid

distributions: the very prominent reduction in overall *Posidonia* canopy height at the low pH sites as a consequence of intense grazing from the fish *Sarpa salpa* (Deudero et al. 2008), compared to lower density long-leaved shoots in the control pH sites (Donnarumma et al. 2014, Scartazza et al. 2017). The increased grazing pressure under highly acidified conditions could explain the decreased spirorbid abundance, as fish grazing removes epiphytic invertebrates (Deudero et al. 2008). Additionally, this variation in the *Posidonia* canopy may indirectly be related to the observed low pH–originating *Simplaria* sp.'s fast juvenile growth. As Spirorbinae are small filter feeders that spend the majority of their lives inside tubes permanently attached to a substrate (Gee 1964, Potswald 1968, Tanur et al. 2010), the organisms in low pH/intense grazing may be rapidly maturing as a response to host plant phenology, a feature that has been highlighted for other *Posidonia* epiphytes (Piazzi et al. 2015).

In addition to predation, it is possible that there are biological interactions between the two species, *Simplaria* sp. and *P. militaris,* which are responsible for their distributions. They may be competing with each other for available space or food, or may have different water movement requirements (Beckwitt 1980; Terlizzi et al. 2000). These factors may be contributing to the relative success of *P. militaris* in the north, compared to its limited southern abundance. The northern sites are more exposed to open water and dominant winds (from north and north-west), whereas the southern sites are within a small bay with less water movement (Rodolfo-Metalpa et al. 2010). This could mean that *Simplaria* sp. populations are better suited to live in more sheltered conditions, or that they are able to fill a niche where conditions are less stable due to pH. A parallel example is provided by the differential occurrence of two non-calcifying polychaete sister species,

Platynereis dumerilii and *Platynereis massiliensis*, around the Ischia CO_2 vents, where the ecological exclusion of *P. dumerilii* in the high CO_2 areas appears to be explained by differences in physiological and life history traits (Lucey et al. 2015). For the spirorbids, a more complete trait analysis able to encompass the full relevant trait space (i.e. testing population samples of each species from all sites and a broader array of traits) would be useful to elucidate which factors are most relevant to explain OA resistance phenotypes (Laughlin & Messier, 2015).

5 Conclusions

This study aimed to identify if and how fecundity, settlement, and juvenile survival were associated with low pH in order to better understand which life-history traits may have an advantage in future marine environments. We found that traits associated with low pH tolerance included increased reproductive output, rapid larval settlement, and high juvenile survival rates. By association, we infer that species with similar life history traits may be better suited to live in future OA inflicted environments, potentially driving future biodiversity patterns. Overall, this study shows how it is possible to guide future research and better our predictive ability of future marine life under increasing ocean acidification by incorporating aspects of community ecology with trait biology.

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7 Figure Captions

Figure 1- Map of sampling sites (black dots) along the two pH gradients of the Castello Aragonese on Ischia Island (Naples, Italy), with southern and northern sites depicted by 'S' and 'N', respectively, and with 'C' indicating control pH, '2' low pH and '3' extreme low pH. Corresponding seawater carbonate data for each site is in Table 1 and all sites are in 3 m *Posidonia* seagrass meadows.

Figure 2 - Mean abundance of spirorbids sampled from south sites (SC, S2, S3) and north sites (NC, N1, N2), colored in red and gray respectively, and with 'C' indicating control pH, '2' low pH and '3' extreme low pH: (A) Total spirorbid abundance (all species combined) (B) *Simplaria* sp. abundance and (C) *P. militaris* abundance, with non-matching lowercase letters indicating significant differences among sites and S.E. as error bars.

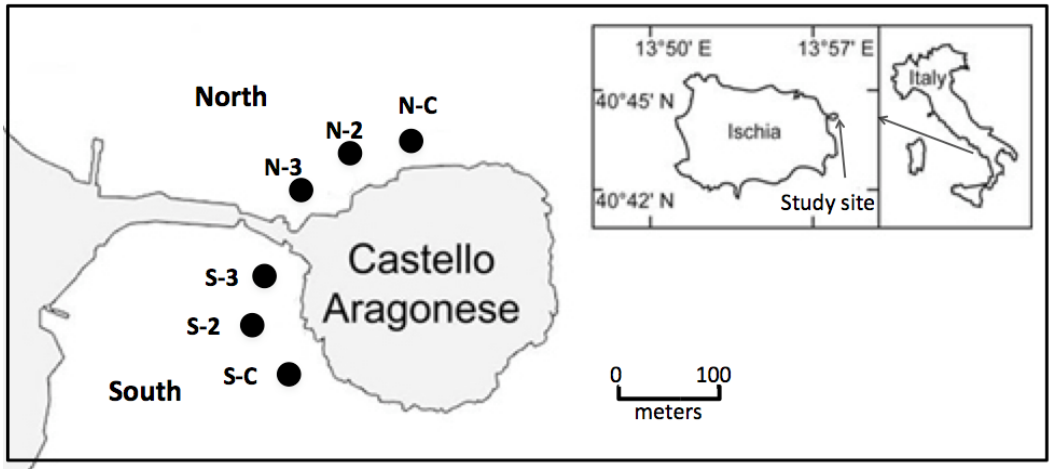
Figure 3 – Total abundance of all spirorbids as they are related to *Posidonia* shoot density: mean number of spirorbids calculated as total species sampled *per* replicate plot area, multiplied by shoot density (m^2), with S.D. as error bars.

Figure 4 - Trends in spirorbid species mean abundance (A) *P. militaris* and (B) *Simplaria* sp. Black dots: mean number of individuals found in each replicate along the northern gradient. Red dots: mean number of individuals found in each replicate along the southern gradient. Black lines are the smoothers for each gradient side; red and gray bands along smoother lines are 95 % CIs.

Figure 5- Fecundity traits and offspring survival from *Simplaria* sp. and *P. militaris* parents cultured in low and control pH conditions respectively, to match their field-originating pH values (7.6 and 8.1); purple and blue bars respectively. (A) Brood size is expressed as the mean number of offspring in the first brood release, (B) mortality as a percent of the beginning brood dead 7 d after initial brood release, and (C) settlement success as the percent of metamorphosed living offspring from each brood 1 day after brood release, (D) total survival as the mean number of offspring living 14 d after the initial brood release, plus any additional offspring released during the 14 d of exposure. Error bars show S.E.; each trait had significantly different means ($p < 0.05$) between species groups.

Figure 6 - (A) *Simplaria* sp. operculum containing embryos: embryonic calcified glands are indicated by white arrows (scale 0.5 mm), (B) a competent trochophore larvae from a *Simplaria* sp. mother (scale 0.1 mm)

740 **8 Figures (low definition)**



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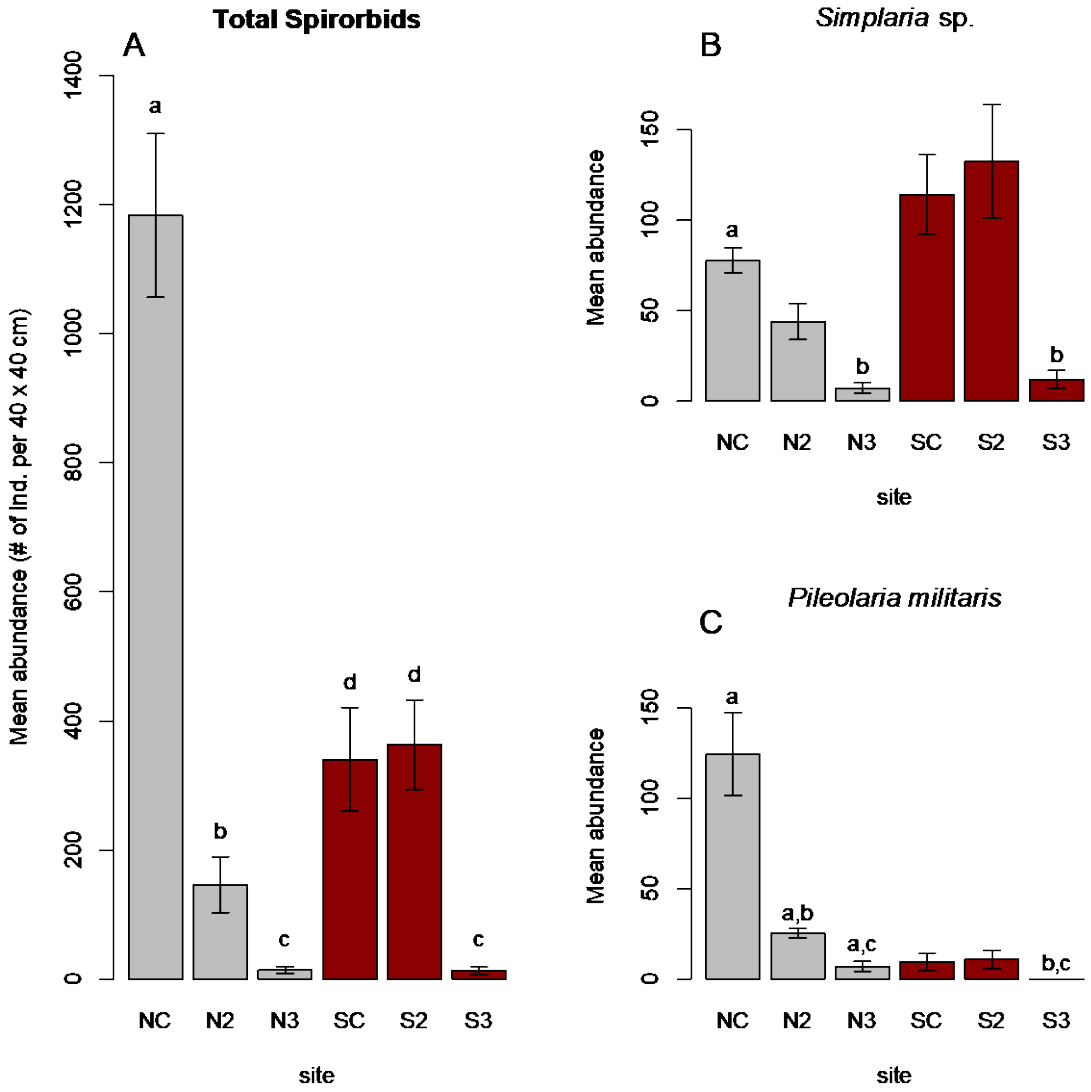

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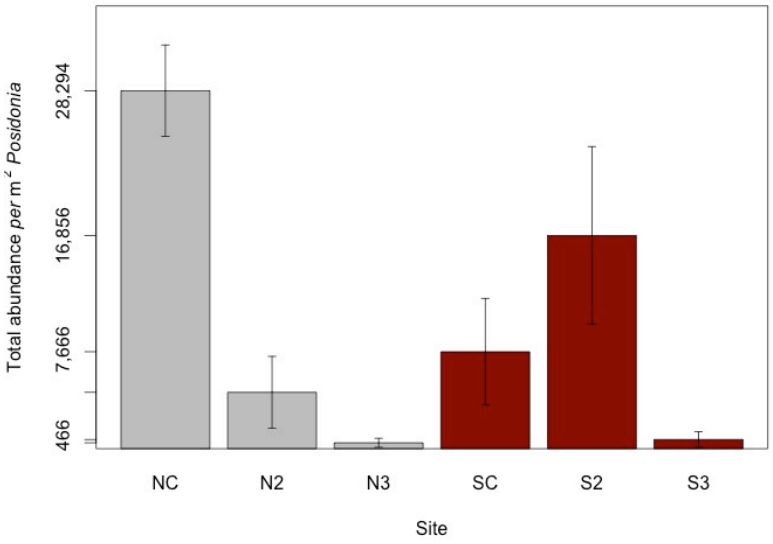

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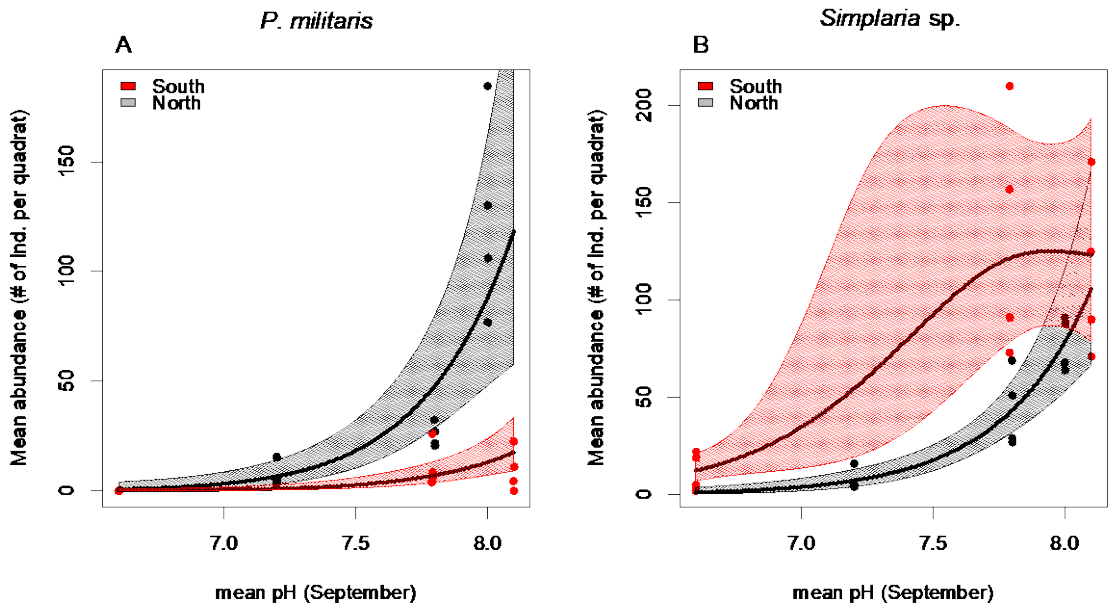

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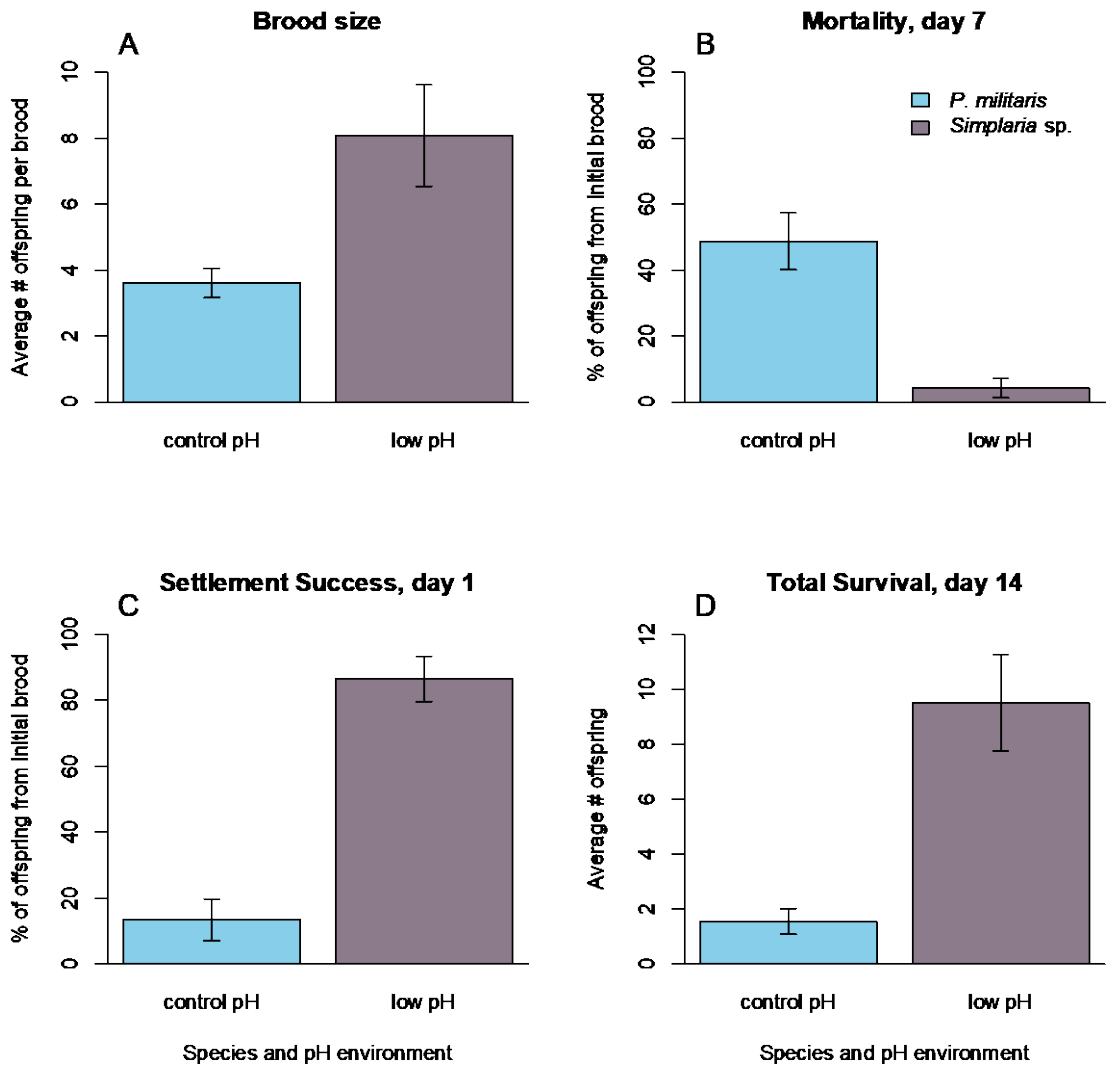

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755 Figure 5

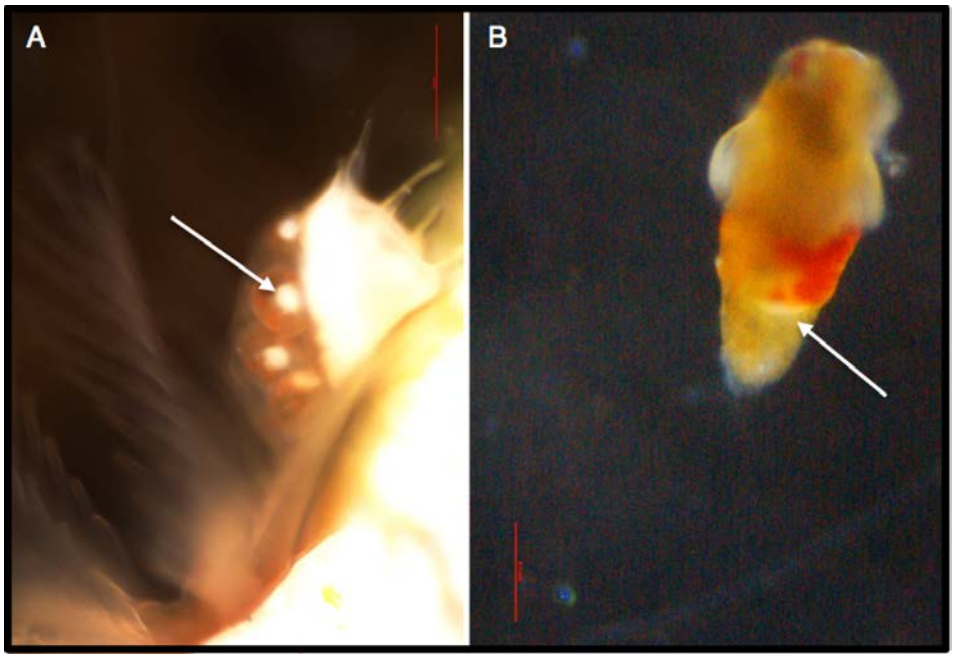

Figure 6

9 Tables

Table 1 Seawater physico-chemical parameters from each pH site (mean ± SD); averaged from a published compilation of six time-series datasets between 2008-2015 in Ricevuto et al. (2014).

Station	mean pH	pCO_2(µatm)	Ω aragonite	Ω calcite	A_T (equival kg^{-1})
Extreme low, S3	6.99 ± 0.34	8830.87 ± 1942.55	0.75 ± 0.50	0.99 ± 0.65	2499.83 ± 23.99
Low, S2	7.61 ± 0.26	2031.19 ± 1,411.65	1.49 ± 0.61	2.52 ± 0.95	2523.68 ± 9.66
Control, SC	8.03 ± 0.08	455.61 ± 94.01	3.36 ± 0.34	5.17 ± 0.47	2499.35 ± 6.94
Extreme low, N3	7.39 ± 0.25	4302.71 ± 5769.22	1.41 ± 0.71	1.94 ± 0.96	2549.45 ± 25.26
Low, N2	7.65 ± 0.29	2639.82 ± 7993.29	2.07 ± 0.70	2.91 ± 1.23	2514.49 ± 7.76
Control, NC	8.03 ± 0.05	468.21 ± 63.85	3.41 ± 0.20	5.20 ± 0.28	2499.67 ± 4.68

765 Table 2. Seawater physico-chemical parameters (a) at the field collection sites, and (b)
766 corresponding laboratory trial pH treatments (mean + SD), measured (in bold) or
767 calculated using the SeaCarb program* over the total trial period for each habitat, either
768 daily (d) or monthly (m). pH is reported using the total scale.

	Control pH (SC)	Low pH (S2)
(a) Field site data		
pH_T	8.04 ± 0.09	7.84 ± 0.24
Temperature (°C)	23.4 ± 0.7	23.8 ± 0.7
Salinity	37.9 ± 0.3	37.9 ± 0.3
A_T (µmol kg^{-1})	2563 ± 3	2560 ± 7
pCO_2 (µatm)	567 ± 100	1075 ± 943
C_T (mol kg^{-1})	0.002 ± 1.02E-04	0.002 ± 1.72E-04
Ω calcite	4.75 ± 0.53	3.52 ± 1.11
Ω aragonite	3.13 ± 0.35	2.32 ± 0.73
(b) Laboratory trials		
pH_T *(days)*	8.08 ± 0.47	7.54 ± 0.53
Temperature (°C) *(d)*	22.31 ± 0.57	22.17 ± 0.83
Salinity *(d)*	36.38 ± 2.11	36.67 ± 2.87
A_T (µmol kg^{-1}) *(m)*	2350.71 ± 53.70	2291.53 ± 122.55
$[CO_2]$ (mol kg^{-1})	9.65E-06 ± 3.10E-06	2.11E-05 ± 6.62E-06
pCO_2 (µatm)	327.88 ± 108.21	721.73 ± 228.33
$[HCO_3^-]$ (mol kg^{-1})	0.002 ± 8.38E-05	0.002 ± 1.57E-04
$[CO_3^{2-}]$ (mol kg^{-1})	2.49E-04 ± 4.75E-05	1.42E-04 ± 2.55E-05
C_T (mol kg^{-1})	0.002 ± 4.601E-05	0.002 ± 1.47E-04
Ω calcite	5.82 ± 1.07	3.33 ± 0.60
Ω aragonite	3.82 ± 0.70	2.19 ± 0.39

769 * Note: Lavigne & Gattuso 2013.
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771

10 Supplementary materials

Site Details:

The north is relatively exposed to the dominant northwestern winds, and the south is a bay-protected area. The venting area in the south is approximately 3000 m^2 and gases are emitted at a rate of 1.4 x 10^6 L d^{-1}. In the north, the area is only 2000 m^2 and the venting rate is slightly decreased at 0.7 x 10^6 L d^{-1} (Hall-Spencer et al. 2008). Specific quantities of the emitted gases are comprised of the following: 90-95 % CO_2, 3-6 % N_2, 0.6-0.8 % O_2, 0.2 - 0.08 % CH_4, and 0.08-0.1 % Ar. No sulfur is present. Although neither seasonal, tidal nor diurnal variation in gas flows have been recorded, the pH does not stay static due to variable bubbling intensity, and shows quite variable values in relatively short, hourly time frames (Kroeker et al. 2011).

Site name and description:	Site GPS Coordinates:
SC: South Control	40.729467, 13.964260
S2: South Low pH	40.730075, 13.963651
S3: South Extreme Low pH	40.731148, 13.963211
NC: North Control	40.732777, 13.965218
N2: North Low pH	40.732316, 13.964464
N3: North Extreme Low pH	40.732000, 13.963716

Laboratory Trial Transport Details:

Samples were transported from field sites by boat to the Villa Dohrn-Benthic Ecology Center and maintained inside 10 L coolers with fresh seawater from each of the collection sites. Samples were kept in seawater matching the pH level of their respective field origin; leaves were inspected to select for living spirobids, and cut in smaller portions to facilitate transport to the ENEA Laboratory in La Spezia, Italy. Transport containers were prepared with spirorbids and unfiltered seawater (volume = 1300 mL; T = 21.96 ± 1.29 °C; pH: control = 8.03 ± 0.08, low = 7.61 ± 0.26; S = 36; density = approx. 100 individuals *per* container) and kept in styrofoam coolers packed with ice to maintain a consistent water temperature. During the 8 h transport to ENEA, temperature and pH were recorded twice using a pH meter with integrated thermometer (SG2, Mettler-Toledo Analytical, Milan, Italy). The mean pH in the containers remained at 8.03 (control samples), or increased from 7.61 to 8.01 (low pH samples). The temperature decreased from 21.96 to 19.00 °C for 1 h in all containers. On arrival at the ENEA laboratory, containers were immediately placed in pre-conditioned temperature baths (T = 22.00 °C, S = 36). Temperature was controlled *via* two thermal baths connected to a temperature conditioner (TR 15, TECO, Naples, Italy) with heaters (V2-Them 300, São Julião do Tojal, Portugal). To enhance a homogeneous mixing of the water, and thus thermal stability of the system, submersible circulation pumps (Aquapump HJ-311, Mondial fauna, Milan, Italy) were also used. Containers were aerated with either ambient (control) air (pCO_2 ~ 380 μatm, for pH = 8.22), or CO_2-enriched air (pCO_2 ~ 1000 μatm, for pH = 7.70). CO_2 gas was slowly released into a Buchner flask to enable mixing using a CO_2 regulator (6000 CO_2, BOC, La Spezia, Italy).

Taxonomy Details:

The tubes and operculum of both the adults and juveniles of the *Simplaria* sp. specimens found in this study closely resemble that of *Simplaria pseudomilitaris* (Thiriot-Quiévreux, 1965), a taxon first described in Villefrance sur Mer, France, and later identified in the Gulf of Naples by Harris (1968), although reported as *Spirorbis berkeleyana* (Rioja, 1942) (Knight-Jones et al. 1974), and in the fouling inside Port of Ischia, Italy (Terlizzi et al. 2000). Morphological similarities of the two are their sinistral coiled (clockwise) tube orientation, similar tube diameter (between 1.5-2 mm), latitudinal tube ridges, and 2-3 indistinct longitudinal tube ridges. The operculum also has a single opercular plate with ornamentation (protuberances, or spines, projecting from top of operculum). The operculum has been described having an elliptical cap with a partially encircling distal papillated rim that is absent on the substratum side (see also Bianchi 1981), yet this feature is not in agreement with this study's specimens, where the rim completely surrounds the distal papillated rim. This feature is, however, in better agreement with a description of *S. pseudomilitaris* from the west coast of the USA made by Beckwitt (1981), who further noted the high variation in operculum morphology in the species. The primary trait that is found in the *Simplaria* sp. specimens of this study that is not in agreement with the *S. pseudomilitaris* descriptions from the literature is the extent of 'ornamentation' on the operculum plate (e.g. Bianchi 1981; Fig. 6).

The morphology of the *Simplaria* sp. here also closely matches *Pileolaria quasimilitaris* with respect to larval and operculum morphology, a taxon first described in the Caribbean Sea (Bailey 1970). In particular, *P. quasimilitaris* has distally projecting calcareous spines on the operculum that form a complete crown. However, it still does

not completely agree as there are up to three indeterminate rows of long, slender spines observed in the operculum crown center in this study's specimens *versus* the two rows of spines originally described for *P. quasimilitaris*. Two other difference between these two species are in the tubes and chaetae: this study's specimens have latitudinal ridges and 2-3 indistinct longitudinal ridges, and no sickle chaetae on the third thoracic fascicles, *versus* the many longitudinal ridges and knobs of *P. quasimilitaris* and presence of these sickle chaetae.

The key taxonomic feature for the genus *Pileolaria* is the presence of sickle chaetae on the third thoracic fascicles (Knight-Jones et al. 1974). In the sister genus *Simplaria* erected by Knight-Jones (1984), all of the characters of the genus *Pileolaria* are found, except the sickle chaetae in the third thoracic fascicles. After examining over 40 *Simplaria* sp. specimens from this study, no sickle chaetae were found. In the original description of *P. quasimilitaris* by Bailey (1970) the chaetae of the third thoracic fascicle are defined as "hooked" chaetae. The morphology of sickle chaetae, is, however, quite variable (Knight-Jones & Fordy 1979) and "hooked" chaetae, *sensu* Bailey (1970) can be considered as sickle chaetae. Regardless, the specimens of this study also lacked hooked chaetae in the third thoracic fascicles. Therefore this relevant character, sickle chaetae absence, leads us to exclude the attribution to our specimens to any other *Pileolaria* species with spines on the operculum (e.g., as *P. semimilitaris*, Vine et al. 1972), and consider our taxon as a member of the *Simplaria* genus, and be considered or a morphotype/ecotype of *S. pseudomilitaris* having more abundant, longer, pronounced distally projecting calcareous spines covering the operculum plate, or a new species from the genus of *Simplaria*. The opercular morphology is a character quite variable in this

854 species, which has lead also to confusion of *S. pseudomilitaris* with other species (e.g.
855 *Spirorbis regalis* in Bailey and Harris (1968)). Analysis of additional *Simplaria*
856 *pseudomilitaris* material from both type locality and other areas could help to account for
857 the possible variability in opercular morphology, however only a genetic analysis would
858 help to determine the correct species status. Yet this is beyond the scope of this study.
859 Therefore, the specimens of this study are designated as *Simplaria* sp.

860 *Supplementary Tables*

861 Table S.1 Number of spirorbids identified in each site replicate, and the ratio of
862 specimens identified (ID'ed) at the species level to the total number of specimens found.
863 These ratios were used to calculate the number of *Simplaria* and *P. militaris* in each
864 replicate, as complete identification of each specimen was not possible due to lost
865 taxonomic features.

Site	Repli-cate	Total spp.	*P. militaris*	*Simplaria* sp.	Other spp.	ID'ed	Ratio ID'ed	*Simplaria* sp. %
NC	*A*	146	62	48	13	123	0.84	39%
	B	178	68	54	12	134	0.75	40%
	C	161	26	32	12	59	0.37	54%
	D	243	78	29	5	112	0.46	26%
N2	*A*	50	12	16	0	28	0.56	57%
	B	80	19	33	2	54	0.68	61%
	C	54	25	35	1	61	1.13	57%
	D	94	20	54	1	75	0.8	72%
N3	*A-D*	46	5	5	0	10	0.22	50%
SC	*A*	113	4	28	3	35	0.31	80%
	B	71	1	25	0	25	0.35	100%
	C	132	0	57	2	59	0.45	97%
	D	180	5	78	5	83	0.46	94%
S2	*A*	75	2	39	1	42	0.56	93%
	B	106	9	42	3	54	0.51	78%
	C	234	1	99	3	103	0.44	96%
	D	158	0	41	1	42	0.27	98%
S3	*A-D*	47	0	13	0	13	0.28	100%

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